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DIFFERENT SUSCEPTIBILITY OF CERVICAL KERATINOCYTES CONTAINING HUMAN PAPILLOMAVIRUS TO CELL-MEDIATED CYTOTOXICITY

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Objective. To detect the factors responsible for the susceptibility of cervical keratinocytes infected with human papillomavirus (HPV) to non-specific lysis mediated by natural killer (NK) and lymphokine activated killer (LAK) cells.

Materials and Methods. Five cervical keratinocyte lines: CaSki, SiHa, HeLa (representing high grade squamous intraepithelial lesion (HSIL)), W12 (representing low grade squamous intraepithelial lesion (LSIL)) and NCx, (normal cervix) were used as target cells in the four-hour lactate dehydrogenase (LDH) release cytotoxicity assay. The effector cells were NK and LAK. The modulatory effects of interferon gamma (IFNγ) and tumor necrosis factor α (TNFα) pretreatment of keratinocytes were investigated by adding IFNγ or TNFα into the flasks of target cells 48 hours before the cytotoxicity assays. The blocking effects of anti-intercellular adhesion molecule-1 (ICAM-1) and anti-lymphocyte function-associated antigen-1 (LFA-1) monoclonal antibodies (Mabs) were also studied.

Results. All the 5 cervical keratinocytes were susceptible to LAK, but not to NK. The sensitivity varied among the cell lines. LAK had better killing effects on HSIL than on LSIL. Pretreatment of target cells with IFNγ and TNFα increased the killing mediated by LAK, but had little effect on NK activity. Anti-ICAM-1 and anti-LFA-1 Mabs inhibited LAK-mediated cytotoxicity.

Conclusions. All the HPV infected keratinocytes used in the experiments are NK-resistant and LAK-sensitive cells. IL-2, IFNγ and TNFα play some critical roles in the regulation of the susceptibility of cervical keratinocytes, especially HSIL to LAK-mediated cytotoxicity in vitro.

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Cervical carcinoma is one of the most common malignant tumors in women worldwide. Human papillomavirus (HPV) infection has been confirmed to be an important etiological agent for cervical intraepithelial lesion and invasive cancer. The local immune response within the HPV infected lesions may be related to the clinical outcome.

It has been shown that cervical keratinocytes infected with HPV16 are able to bind peripheral blood lymphocytes (PBLs) activated by IL-2.[1] The binding was increased by interferon gamma (IFNγ) pretreatment of target cells in a dose-dependent manner. It could be inhibited by anti-intercellular adhesion molecule-1 (ICAM-1) and anti-lymphocyte function-associated antigen-1 (LFA-1) monoclonal antibodies (Mabs), suggesting that ICAM-1 may be a critical adhesion molecule in the interaction.

In this study, we extended these observations by examining 1) the susceptibility of cervical keratinocytes to non-specific lysis mediated by natural killer (NK) and lymphokine activated killer (LAK) cells; 2) the modulatory effects of IFNγ and TNFα (tumor necrosis factor α) pretreatment of keratinocytes, and 3) the blocking effects of anti-ICAM-1 and anti-LFA-1 antibodies. Lysis was quantified by a non-isotopic method based upon the colorimetric detection of lactate dehydrogenase (LDH) released from the lysed cells in a four-hour cytotoxicity assay.

MATERIALS AND METHODS

Preparation of lymphocytes. Buffy coats from

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normal human volunteers were obtained from Cambridge Blood Transfusion Centre. Lymphocytes were isolated by density gradient centrifugation on Histopaque (density: 1.083). After washing twice in $RPMI_{1640}$ medium, PBLs were cultured overnight at $2\times 10^3/L$ in $RPMI_{1640}$ containing 20% Fetal Calf Serum (FCS) at 37℃ in a 5% CO_2, 95% air atmosphere. After removing adherent cells, the NK activity of these lymphocytes was assessed. Activation of LAK cells was achieved by culturing in the above-mentioned medium containing 100 units/ml of IL-2 (British Biotechnology) for four days.

Target cells. We selected 5 cervical keratinocyte lines which were well or poorly differentiated and infected with different subtypes of HPV or different copies of the virus in order to observe if there was any relationship among them.

Cervical carcinoma derived keratinocytes, CaSki (HPV16+, 300–500 copies), SiHa (HPV16+, 1 copy) and HeLa (HPV18+, 100 copies), were maintained in continuous culture in Glasgow's modification of Eagle's medium (GMEM) containing 10% FCS at 37℃ in a 5% CO_2 incubator. They were used as models of high grade squamous intraepithelial lesion (HSIL) according to the Bethesda system.[2]

W12, as a model of low grade squamous intraepithelial lesion (LSIL), is a cervical keratinocyte line which is immortalised but non-transformed by natural infection with HPV16.[3] It was derived from non-malignant cervical tissue which was diagnosed colposcopically and histologically as LSIL. It contained about 100 copies of HPV16 DNA in the episomal form. Cell culture was performed using lethal irradiated Swiss G3T3 mouse fibroblast feeder cells and GMEM medium containing epidermal growth factor (EGF) at a concentration of 10 mg/L and 10% FCS.

Normal ectocervical keratinocytes (NCx) were obtained from hysterectomy specimens, in which there was no abnormal finding morphologically (all patients had recently documented normal cervical smears). They were confirmed to be HPV-negative by PCR.

K562 cell line was derived from a patient with chronic myelogenous leukemia in blast phase. It was used as a known NK sensitive cell line. The cells were cultured in $RPMI_{1640}$/10% FCS medium.

Pretreatment of target cells. IFNγ (Genzyme) was added to the target cells at a concentration of 300 units/ml 48 hours before the cytotoxicity assays. TNFα (British Biotechnology) was used to treat the target cells in a dose of 200 units/ml 48 hours prior to the experiments.

Cytotoxicity assay. An aliquot of the desired target cell line was removed by trypsinization. After washing, the cells were resuspended in $RPMI_{1640}$ /2% bovine serum albumin (BSA) with the dilution of $10^2/L$. Effector cells in defferent dilutions (2×10^2–$5\times 10^3/L$) were introduced into 96-well round-bottomed microplates in 50 μl of $RPMI_{1640}$/2% BSA. Target cells were added in a volume of 50 μl of $RPMI_{1640}$/2% BSA at a dilution of $10^2/L$ with or without pretreatment. After incubating at 37℃ in a 5% CO_2, 95% air incubator for 4 hours, the plates were centrifuged at a low speed for 3 minutes, after which, 50 μl of the co-incubated cell supernatant was transferred into a flat-bottomed plate. Reagents in CytoTox 96™ Non-Radioactive Cytotoxicity Assay Kit (Promega) were used according to the manufacturer's instructions. The absorbance was recorded at a wavelength of 490 nm on an Elisa reader (Dynatech MR5000). The percentage of cytotoxicity was calculated with the formula given by the manufacturer:

$$\%.\,\text{Cytotoxicity} = \frac{\text{Experimental OD} - \text{Effector spontaneous OD} - \text{Target spontaneous OD}}{\text{Target maximal OD} - \text{Target spontaneous OD}} \times 100\%$$

Blocking assay. Anti-ICAM-1 (British Biotechnology) monoclonal antibody (Mab) was added to target cells at 37℃ at the concentration of 20 mg/L 30 minutes before addition of lymphocytes. Anti-LFA-1 Mab (British Biotechnology) was added to lymphocytes at 37℃ at the concentration of 10 mg/L 30 minutes before the co-culture assay. Anti-HLA.ABC and anti-HLA.DR Mabs (Dako)

Chinese Medical Journal, 109(11): 854–858, 1996.

at the concentrations of 0–40 mg / L were also used in an attempt to block the cell lysis.

RESULTS

There was some variation among the results from different donors. The representative graphs shown in Figs. 1–4 were selected from at least 3 repeated experiments.

The cytotoxicity mediated by freshly isolated PBLs on CaSki, SiHa and K562 (Fig. 1). After PBLs were isolated and cultured in vitro overnight without IL–2 pretreatment, they were used as NK cells. NK cells were effective to kill K562 cells but had little effect on CaSki and SiHa with or without IFNγ pretreatment. NK did not show cytolytic effects on W12 and NCx (data not shown).

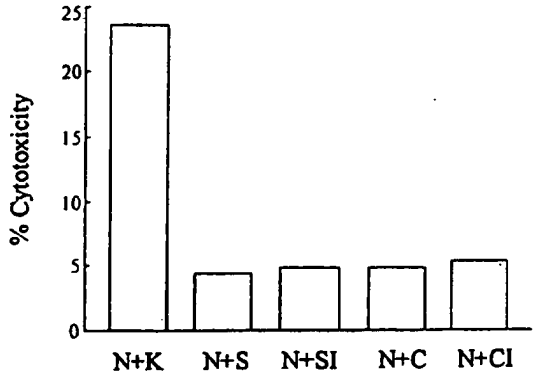

Fig. 1. The cytotoxicity mediated by NK (N) to K562 (K), SiHa (S) and CaSki (C). SI: SiHa pretreated by IFNγ (I); CI: CaSki pretreated by IFNγ (I). E:T (effector:target) = 10:1.

The cytotoxicity produced by LAK on CaSki SiHa, HeLa, W12 and NCx (Fig. 2). It was shown that LAK cells had a broader range of target cell destruction. The activity was effector:target (E:T) ratio–dependent and cell line–dependent. Satisfactory cytotoxicity was produced at an E:T ratio of 10:1 or more. The cytolytic effects on HeLa, CaSki and SiHa were better than those on W12 and NCx.

The effects of IFNγ and TNFα pretreatment of target cells on cytotoxicity (Fig. 3). IFNγ was able to upregulate the susceptibility of HSIL (SiHa, CaSki and HeLa) to LAK. Pretreatment of target cells

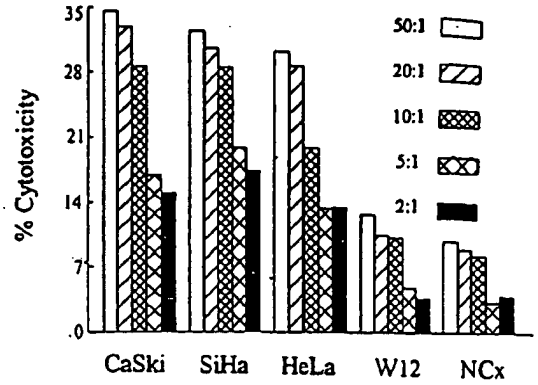

Fig. 2. The cytotoxicity produced by LAK to CaSki, SiHa, HeLa, W12 and NCx with different E:T ratios.

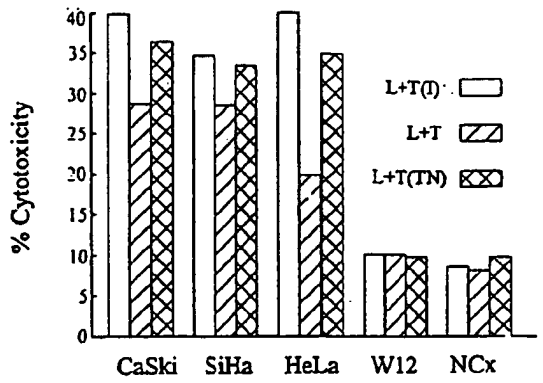

Fig. 3. The effects of IFNγ (I) and TNFα (TN) pretreatment of target cells (T) on cytotoxicity. E:T = 10:1.

with TNFα also improved the killing mediated by LAK.

The blocking effects mediated by anti–ICAM–1, anti–LFA–1, anti–HLA.ABC and anti–HLA.DR Mabs (Fig. 4). Both anti–ICAM–1 and anti–LFA–1 Mabs were able to block the cytotoxicity. Ten mg / L for anti–ICAM–1 and 5 mg / L for anti–LFA–1 Mabs were optimal for the blockage. But no similar result was found in all cases of preincubation with anti–HLA.ABC and anti–HLA.DR Mabs.

DISCUSSION

In the study of immune surveillance mechanisms in virus infection and neoplastic transformation, a growing body of evidence suggests that NK cells are involved in the defense against viral infection and in the destruction of human neoplastic cells without prior sensitization.[4] Although the killing

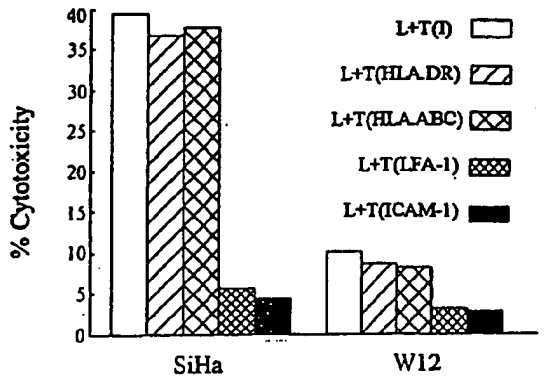

Fig. 4. The blocking effects produced by Mabs on cytotoxicity mediated by LAK to target cells. E:T = 10:1.

effect is antibody-independent and non-MHC restricted, the targets for NK are very limited. It has been reported that HPV16-immortalized cervical carcinoma cell line (QGU) is resistant to natural killing of NK cells, but sensitive to LAK cells.[5] In our experiments, freshly isolated PBLs were effective to lyse K562 but minimally responsive to CaSki and SiHa whether with or without IFNγ pretreatment of target cells, suggesting that CaSki and SiHa were NK resistant.

LAK cells are similar to NK cells in morphology but more effective than NK in mediating cytotoxicity. The target cells include both NK-sensitive and NK-resistant cells, which are not HLA-restricted and not limited to tumor cells. In our report, LAK activity against cervical keratinocytes was shown to be E:T ratio-dependent and cell line-dependent. LAK showed better killing effects on HSIL than on LSIL. It seemed that the susceptibility was correlated with neither the subtype of HPV infection nor the copies of the virus. So we think it is the poor differentiation that makes the cells more sensitive to LAK mediated killing.

It has been suggested that the interaction of LFA-1 / ICAM-1 is the principal pathway of T cell adhesion to target cells.[6] LFA-1 is expressed by all leukocytes, and is a member of the integrin family. Its counter receptor on target cells is ICAM-1. The expression of ICAM-1 on the surface of both hematopoietic and non-hematopoietic cells is selectively induced or increased within hours by cytokines such as IFNγ and TNFα. In another report, HSIL showed strong expression of ICAM-1 on full thickness of epithelium, while in LSIL, ICAM-1 expression was limited to the basal layers of epithelium.[1]

To understand the LFA-1 / ICAM-1 adhesion effect in the cytotoxicity, we incubated target with anti-ICAM-1, and LAK with anti-LFA-1 Mabs. We found that the antibodies could effectively block the killing effect in both IFNγ pretreated and untreated targets. However, no overall blocking effect in cytotoxicity has been found in cases using anti-HLA.ABC and anti-HLA.DR Mabs preincubation. The results are consistant with the findings that anti-ICAM-1 and anti-LFA-1 Mabs blocked in vitro lymphocyte:keratinocyte adhesion, while anti-HLA.ABC and anti HLA.DR Mabs had no such blocking effect.[1]

There are contradictory results in the published data about the effectiveness of IFNγ on target cell susceptibility to lysis by LAK. Some studies indicated that IFNγ treatment of certain tumor cells increased their susceptibility to LAK,[7] while others showed no change or even protection of these cells from lysis.[8] Our results demonstrated that IFNγ upregulated the targets' susceptibility to LAK, especially HSIL. We consider that the increase may be partially attributed to ICAM-1 antigen.

TNFα possesses a wide range of biologic activities,[9] one of which is the ability to mediate cytotoxicity both in vitro and in vivo. Some reports showed that most cells derived from cervical malignancies are resistant to the direct cytolytic effects of TNFα.[10] In our assays, we observed that TNFα and IFNγ showed similar effects on the cytotoxicity mediated by LAK. Perhaps it was because that TNFα and IFNγ shared some biological activities and they were positively interacted in many systems. TNFα promoted the systhesis of IFNγ and this could explain the increased sensitivity of target cells to LAK lysis.

In conclusion, our results indicated that IL-2, IFNγ and TNFα played some critical roles in the regulation of the susceptibility of cervical keratinocytes, especially HSIL to LAK-mediated cytotoxicity in vitro. HSIL cells infected with HPV showed the best susceptibility to LAK-mediated

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cytotoxicity. Whether these factors are relevant to clinical application is under investigation.

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Effect of Stapled Anastomosis in Surgery of the Esophagus-experience of 1605 Cases. Sun Yue, et al. Dept Thorac Surg General Hospital of PLA, Beijing 100853. Chin J Thorac Cardiovasc Surg 1996; (12)1:20.

Leakage at the anastomotic site in esophageal surgery remains an annoying problem to both patients and thoracic surgeons. Many papers have reported the advantages of using the stapling device in esphageal anastomosis. In order to reveal the merits of stapling anastomosis in exophageal surgery, the authors summarized the experience of stapling anastomosis in the resectional treatment for carcinoma of the esophagus and gastric cardia on 1605 cases during the period from August 1989 to February 1994. There were 1281 males and 324 females with a ratio of 3.95:1. Their age ranged from 28 to 81 years with 1184 patients (73.80%) belonging to the 50–69 age group. Carcinoma of the esophagus was present in 1044 patients and carcinoma of the cardia in 561. The anastomosis was performed in the cervical region in 35 cases and intrathoracically in 1570. Anastomotic leakage occurred in 16 patients with an overall incidence of 1% (16/1605). In comparison, the incidence of anastomotic leakage in the cervical anastomosis group was 14.3% (5/35) vs 0.7% (11/1570) in the intrathoracic anastomosis group. However, the incidence of intrathoracic anastomotic leakage had been as high as 1.4% (8/575) prior to 1986, but thereafter, the incidence came down to 0.3% (3/995) Anastomotic stricture occurred in 16 patients (1%). The clinical experience indicated that stapling anastomosis with a stapling device is a very effective procedure in reducing the incidence of esophageal anastomotic leakage especially in the intrathoracic anastomosis.